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## Bioconcentration, Metabolism and Excretion of Triclocarban in larval Qurt Medaka (*Oryzias latipes*)

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### Abstract

The antimicrobial triclocarban (TCC) is frequently found in personal care products and commonly observed in surface waters and sediments. Due to its long environmental persistence TCC accumulates in sewage sludge. It also shows a high unintended biological activity as a potent inhibitor of the soluble epoxide hydrolase (sEH) and may be an endocrine disruptor. In this study, we investigated bioconcentration, metabolism and elimination of TCC in fish using Medaka (*Oryzias latipes*) as a model. Medaka larvae (7±1 days post hatching) were exposed to 63 nM (20 µg/L) TCC water for 24 hours. The LC-MS/MS analysis of water and tissues provided bioconcentration of TCC and its metabolites in fish body and rapid excretion into culture water. Results from tissue samples showed a tissue concentration of 34 µmol/kg and a log bioconcentration factor (BCF) of 2.86. These results are slightly lower than previous findings in snails and algae. A significant portion of the absorbed TCC was oxidatively metabolized by the fish to hydroxylated products. These metabolites underwent extensive phase II metabolism to yield sulfate and glucuronic acid conjugates. The most abundant metabolite in fish tissue was the glucuronide of 2'-OH-TCC. Elimination of TCC after transferring the fish to fresh water was rapid, with a half-life of 1 hour. This study shows that larval medaka metabolize TCC similarly to mammals. The rapid rate of metabolism results in a lower bioconcentration than calculated from the *n*-octanol/water partition coefficient of TCC.

### Keywords

Triclocarban; triclocarban metabolites; Qurt medaka (*Oryzias latipes*); online-solid phase extraction; liquid chromatography electrospray tandem mass spectrometry; bioconcentration

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## 1. Introduction

Triclocarban (TCC, 3,4,4'-trichlorocarbaniide, CAS 101-20-2) is a common antibacterial agent in personal care products (PCP). Recent studies have indicated unintended biological activity of TCC in animals. Administration of TCC at high concentration along with testosterone significantly enlarges male accessory organs such as seminal vesicles, glans penis and ventral prostate in rat (Chen et al., 2008). Ahn et al. also demonstrated that TCC exposure enhances estradiol-dependent or testosterone-dependent activation of estrogen receptor- and androgen receptor-responsive gene expression in human ovary cells (Ahn et al., 2008). Moreover TCC affected the transcription of genes responding to thyroid hormone in frog and rat cells (Hinther et al., 2011). In the freshwater mudsnail, *Potamopyrgus antipodarum*, TCC exposure promotes embryo production (Giudice and Young, 2010). TCC is also an inhibitor of the soluble epoxide hydrolase (sEH) (Morisseau et al., 2009; Schebb et al., 2011b), an enzyme of the arachidonic acid cascade (Morisseau and Hammock, 2005). The *in vitro* potency of TCC (IC<sub>50</sub> for the human sEH: 24±5 nM) (Schebb et al., 2011b) was comparable to synthetic inhibitors IC<sub>50</sub>s in the low nanomolar range (Schebb et al., 2011a) which affect the biological regulation of inflammation, pain and blood pressure *in vivo* (Imig and Hammock, 2009; Inceoglu et al., 2006; Inceoglu et al., 2011).

Due to its frequent usage, levels of TCC have been detected in surface waters in the United States up to a concentration of 5 µg/L (16 nM) (Halden and Paull, 2004, 2005). The Targeted National Sewage Sludge Survey, published in 2009 by the US Environmental Protection Agency (EPA), reports TCC in all 84 sludge samples analyzed. TCC was found at up to 0.44 g/kg, which indicates a high accumulation of TCC in sludge (EPA, 2009). Some studies also show a bioaccumulation of TCC in aquatic organisms. Investigations of the TCC concentration in water, algae (*Cladophora spp.*) and snail (*Helisoma trivolvis*) collected downstream of a waste water plant demonstrate a significant bioaccumulation factor (BAF) of 1,600–2,700 (log BAF 3.2–3.4) in both species (Coogan et al., 2007; Coogan and La Point, 2008). Higgins et al. (2009) also showed that the freshwater worm *Lumbriculus variegatus* absorbs TCC from sludge. No data is available for most aquatic organisms, including fish, which are vulnerable to exposure with environmental toxins especially in developmental stages. Although bioconcentration in aquatic organisms correlates in many cases well with the physicochemical properties of a compound e.g. the *n*-octanol/water partition coefficient (K<sub>OW</sub>) (Kenaga, 1980a, b), it is modified by competing pharmacokinetic processes, such as metabolism. In mammals, TCC undergoes extensive oxidative metabolism catalyzed by cytochrome P450 monooxygenases (P450) (Baumann et al., 2010). The primary phase I metabolites are monohydroxylated TCC derivatives, in the *ortho* positions to the urea moiety (Fig. 1) (Birch et al., 1978; Hiles and Birch, 1978a, b; Hiles et al., 1978; Jeffcoat et al., 1977; Warren et al., 1978). Phase II metabolism leads to sulfate and glucuronic acid conjugates of these metabolites, which are the main metabolites detected in the plasma and bile of mammals, (Birch et al., 1978; Hernando et al., 2007; Hiles and Birch, 1978a). Additionally direct *N*-glucuronidation of TCC is an important pathway in human TCC metabolism (Birch et al., 1978; Schebb et al., 2011b). These metabolic transformations, summarized in Figure 1, result in efficient elimination from humans because no accumulation of exposed subjects is observed. (Gruenke et al., 1987; Howes and Black, 1976; Schebb et al., 2011b; Taulli et al., 1977).

Fish possess metabolic enzymes similar to mammals: P450, sulfotransferases and uridine diphosphate glucuronosyl transferases (UGT) (James et al., 1994; Tate, 1988). However, for many xenobiotics, the biotransformation processes take place at slower rates than in mammals (Hoffman et al., 1990). This led us to hypothesize that the TCC absorbed by fish will undergo metabolic pathways similar to those previously observed in mammals. In order to test this hypothesis and investigate its effect on the bioconcentration we exposed 7-day

old Japanese Qurt medaka (*Oryzias latipes*) larvae to TCC and analyzed bioconcentration, metabolism and excretion. To the best of our knowledge this is the first study about the metabolic fate and the excretion of TCC in aquatic species.

## 2. Materials and Methods

### 2.1 Chemicals

3,4,4'-Trichlorocarbanilide (Triclocarban, TCC) was purchased from Sigma-Aldrich (St Louis, MO) and contained 0.7 % diclocarban (DCC) as impurity determined by LC-MS. The analytical TCC standard was further purified ( $\geq 99.9\%$ ) by repeated re-crystallization from ethanol and petroleum ether respectively. The internal standard (I.S.) (4-chlorophenyl  $^{13}\text{C}_6$ )-TCC (99%  $^{13}\text{C}$ ) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). The TCC metabolites and analogs, structures shown in Figure 1, were synthesized in house as described previously (Ahn et al., 2008; Baumann et al., 2010). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and were of the highest quality available.

### 2.2 Medaka fish exposure and sampling

Qurt medaka (*Oryzias latipes*) cultured in the Aquatic Health Program at the University of California at Davis were used for the exposure study (Leon, 2007). Medaka eggs were collected from the culture system and maintained in embryo rearing solution (1 g/L NaCl, 0.030 g/L KCl, 0.040 g/L  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 80 mg/L  $\text{MgSO}_4$  and 1 mg/L Methylene Blue in distilled water) until hatching. Female embryos were separated at 3 days post-fertilization based on sex-linked coloration (Wada et al., 1998). After hatching, fish were transferred to clean reconstituted water. Reconstituted water was prepared according to the guidelines of the US Environmental Protection Agency (EPA) (Horning, 1985). The fish were fed with formulated diet twice a day as described elsewhere (DeKoven, 1992). Feeding was stopped two days before the exposure experiments. All experiments were carried out at a water temperature of 25 °C with a photoperiod of 16 h: 8 h (light:dark).

**Bioconcentration study**—In fifteen 500 mL glass beakers, 400 mL of 20  $\mu\text{g/L}$  (63 nM) TCC solutions were freshly prepared by adding a 50 ppm TCC stock solution (160  $\mu\text{M}$ ) in ethanol to reconstituted water. The exposure was started by transferring 50 medaka larvae ( $7 \pm 1$  days post hatching) to each beaker. All fish ( $n=50$ ) in triplicate of beakers were harvested using mesh screen at various time points after exposure initiation (1, 3, 9, 15 and 24 h). The wet weight of the fish was measured and the samples were frozen at  $-80^\circ\text{C}$  until analysis. Additionally, a triplicate of 50 fish each was collected 1 h prior to the exposure serving as control. All the exposures were performed in triplicate and concentrations were calculated as mean  $\pm$  SE. Water samples were collected over the incubation time from three different sets of beakers: i) TCC (20  $\mu\text{g/L}$ ) in clean reconstituted water with 50 fish, ii) TCC (20  $\mu\text{g/L}$ ) in 400 mL of fresh reconstituted water without fish, iii) TCC (20  $\mu\text{g/L}$ ) in 400 mL of used aquarium water from medaka culture system without fish. Water samples (0.5 mL) were collected after 5 min, 1, 3, 6, 9, 12, 15, 18, 21 and 24 h of exposure and stored in  $12 \times 32$  mm amber glass vials (Fisher Scientific) at  $-80^\circ\text{C}$  until analysis. Three independent incubations were carried out for each set and concentrations were calculated as mean  $\pm$  SD.

**Elimination study**—In this experiment, 1,800 fish ( $7 \pm 1$  days post hatching) were exposed with reconstituted water containing 20  $\mu\text{g/L}$  TCC in 4 L beakers in the same fish/water ratio as in the bioconcentration study (2.2.1). After 24 h exposure, fish were distributed into 500 mL beakers (50 fish each) containing 400 mL of clean reconstituted water. All fish in three beakers were harvested at 5 min, 1, 3, 6, 24, 48, 72, 96, 120, 144 and 168 h post transfer to clean water. Feeding was resumed after 120 h sampling. Only living fish (mortality was observed due to starvation, see supplementary data) were harvested and their weight and

number of fish were recorded. Water samples were collected from the same set of three beakers with TCC exposed fish at the same interval as for fish sampling.

### 2.3. Online-SPE-LC-MS/MS analysis of TCC and its metabolites

Online SPE-LC analysis was performed with an optimized method as previously described (Schebb et al., 2011b; Schebb et al., 2010). Details are presented in the supplementary data.

### 2.4 Preparation of standard solutions and sample preparation

Analyte stock solutions (10 mM) were prepared in DMSO and stored at  $-20^{\circ}\text{C}$ . A solution of I.S. was prepared in acetonitrile(ACN)/acetic acid (98/2, v/v) to a final concentration of 20 nM. For calibration, a multi standard solution (100  $\mu\text{M}$  in ACN) of the analytes was prepared from stock solutions and sequentially diluted in water/ACN 50/50 (v/v) containing 10 nM I.S. yielding concentrations of 0.1 pM- 3  $\mu\text{M}$  of each analyte.

For water analyses, 300  $\mu\text{L}$  aliquots of samples were mixed with I.S. solution 1:1 (v:v), vortexed and directly injected (100  $\mu\text{L}$ ) into the online-SPE-LC-ESI-MS/MS system. For tissue analysis, 50 fish larvae (ca. 30 mg) made up to a volume of 200  $\mu\text{L}$  with water assuming a density of the fish of 1 mg/ $\mu\text{L}$ . After addition of 200  $\mu\text{L}$  of I.S. solution and two stainless steel metal balls (i.d. 0.5 mm), the samples were homogenized on a Retsch Mixer Mill MM 302 (Retsch, Haan, Germany) at 30 Hz for 30 min. The resulting suspension was centrifuged at  $16,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min and the supernatant was directly injected (5  $\mu\text{L}$ ) into the online-SPE-LC-ESI-MS/MS system.

### 2.5 Calculation of BCF

The bioconcentration factor (BCF) was calculated by ratio of the TCC concentration at steady state measured in fish tissue compared to the water concentration (eq. 1).

$$\text{BCF} = c_{\text{tissue}} / c_{\text{water}} \quad (\text{eq. 1})$$

## 3. Results

### 3.1 Quantification of TCC and its metabolites

In order to quickly analyze TCC and its metabolites in water and fish tissue samples, we utilized a recently described online SPE-LC-ESI-MS/MS method. This approach provides excellent accuracy, precision and robustness for the direct analysis of biological samples (Schebb et al., 2011b). This method was further improved to detect low concentrations of TCC and its metabolites in water samples. Representative chromatograms for standards and fish tissue and water samples from the medaka exposure study are shown in Figure 2 and details about the method are described in the supplementary data.

### 3.2 Absorption, metabolism and elimination of TCC in medaka fish

**3.2.1 Bioconcentration of TCC**—As shown in Figure 3A, the TCC water concentration of control incubations without fish remained stable over the incubation time of 24 h at  $62 \pm 4$  nM TCC. A decrease of TCC concentration was observed after the addition of fish (Fig. 3A). After 5 minutes of exposure, the TCC water concentration of  $54 \pm 4$  nM was lower than in the control samples and decreased further over the incubation time to  $44 \pm 1$  nM. Fish tissue concentrations increased dramatically over 3 hours from <LOD pre-exposure to  $35 \pm 4$   $\mu\text{mol/kg}$  and remained at a steady state of  $34 \pm 2$   $\mu\text{mol/kg}$  during the 24 h of incubation time (Fig. 3C). Based on the concentration ratio of tissue to water over this steady state period, a log BCF  $2.86 \pm 0.05$  was calculated. For the more polar TCC congener, DCC, present as an

impurity in the TCC preparation used, a log BCF of  $2.44 \pm 0.05$  was calculated accordingly (Supplementary Data, Fig.S1).

**3.2.2 Metabolism of TCC by medaka fish**—Analysis of the water samples during the incubation of fish with 20 µg/L (63 nM) TCC revealed formation of oxidative metabolites and their conjugates. As shown in Figure 3 B, for the oxidative metabolites in water, 2'-OH-TCC and 3'-OH-TCC, the water concentration increased within the 24 h incubation time up to a concentration of  $10 \pm 5$  pM and  $26 \pm 15$  pM, respectively. After 24 h, a third hydroxylated metabolite of TCC, 6-OH-TCC, was detected at a concentration of  $2 \pm 1$  pM. The primary metabolite in water was the glucuronic acid conjugate of 2'-OH-TCC, 2'-O-Gluc-TCC, reaching a concentration of  $280 \pm 1$  pM after 24 h incubation; whereas the sulfate conjugate, 2'-O-SO<sub>3</sub>-TCC was detected at a much lower level of  $7 \pm 2$  pM. Additionally small amounts of oxidative metabolite DHC (Fig.1) were detected as well as an incubation time dependent increase in the 3'-O-Gluc-TCC and 6'-O-Gluc-TCC peak area. No metabolite formation was detected in control water samples of fresh and aquarium water (from medaka culture system) incubated without fish.

In the fish tissue, the concentrations of 2'-O-Gluc-TCC, 2'-O-SO<sub>3</sub>-TCC, 2'-OH268 TCC, 3'-OH-TCC and 6-OH-TCC increased rapidly within 3 h of incubation and continued to increase until 15 h when steady state was reached (Fig. 3D). DHC was also formed reaching levels up to  $419 \pm 40$  nmol/kg after 24 h of incubation. The peak areas for 3'-O-Gluc-TCC and 6-O-Gluc-TCC increased over time suggesting an additional formation of these conjugates. Among the quantified metabolites, 2'-O-Gluc-TCC was predominantly formed (up to a concentration of  $4.7 \pm 0.1$  µmol/kg fish tissue) which is consistent with the observed water concentration. With  $914 \pm 30$  nmol/kg, the 2'-O-SO<sub>3</sub>-TCC was the second most abundant metabolite detected in fish tissue (Figure 3 D).

**3.2.3 Screening of further metabolites**—The medaka fish may form other metabolites not detected by the SRM-MS method. Therefore, fish samples after 24 h incubation with 20 µg/L TCC were screened for further biotransformation products. The LC-ESI(-)-SCAN-chromatogram showed multiple peaks. However, except for TCC, none of the dominating peaks contained the characteristic isotopic pattern of chlorine containing compounds. Therefore, the fish homogenate samples were screened in selected ion monitoring (SIM) on the *m/z* of expected metabolites. No sulfate conjugates of hydroxylated TCC metabolites (*m/z* 409; 411) other than 2'-O-SO<sub>3</sub>-TCC were detected. Moreover, no peaks of dihydroxylated TCC species (*m/z* 345; 347) or other monohydroxylated TCC metabolites at *m/z* 329; 331 were found. However, in the SRM chromatogram of the *m/z* 329/168, an additional peak at 3.0 min is detected (peak X in Fig. 2E), which increased with the incubation time. Because of the low concentration, no fragment spectrum of this peak could be obtained. Thus there is currently no information about the structure of this potential metabolite. In the LC-ESI-SIM chromatograms at *m/z* 295 and 297 an intense peak at 2.2 min was detected. It showed fragment ions at *m/z* 142 and *m/z* 168 corresponding to the aniline and isocyanate fragment of a carbanilide derivative bearing one chlorine atom in one aniline ring and chlorine and a hydroxyl group in the other (Supplementary data Fig. S2).). Therefore, it was assumed that this metabolite is formed by hydroxylation of DCC and is referred to in the following as OH-DCC. However, it should be noted that this compound could also be formed by dehalogenation and subsequent hydroxylation as discussed for the formation of DHC from TCC (Baumann et al., 2010). The OH-TCC peak was absent in control water incubations and unexposed fish, but its area increased in water and fish tissue analysis over the incubation time as described above for the hydroxylated TCC metabolites.

**3.2.4 Elimination of TCC**—After 24 h exposure, the majority of TCC remained in the 400 mL water (92.1%). A significant portion (5.5%) of the compound was absorbed by the



50 fish (total body weight  $28 \pm 5$  mg). Only 1.8% was present as metabolites in fish tissue and as little as 0.6% of the TCC was released as metabolites to the water (Insert in Figure 3C). In order to evaluate the elimination of TCC in more detail, the fish were transferred after 24 h of exposure into clean water. The TCC concentration in fish tissue ( $40 \pm 5$   $\mu\text{mol/kg}$ , total amount  $1.3 \pm 0.2$  nmol) rapidly decayed with an initial  $t_{1/2}$  of 1 h and after 48 h only 0.1 % of the initial concentration of TCC ( $44 \pm 2$  nmol/kg; total amount  $1.5 \pm 0.1$  pmol) remained in the fish (Fig. 4C).

The TCC water concentration increased quickly in 3 h to  $93 \pm 2$  pM and then slowly declined over time to  $30 \pm 16$  nM after 168 h (Fig. 4A). The log BCF calculated from the ratio of fish TCC concentrations (24–44 nmol/kg) and water concentration (30–60 pM) between 48 h and 168 h was  $2.78 \pm 0.13$  and thus consistent with the value found in the absorption study (see section 3.2.1).

In the first hours after removal from exposure beakers, the concentration of the metabolites 2'-OH-TCC, 3-OH-TCC and 6-OH-TCC, DHC, 2'-O-SO<sub>3</sub>-TCC and 2'-O-Gluc-TCC in fish tissue increased and reached the highest levels after 3 h. Thereafter, the levels slowly decreased with a  $t_{1/2}$  around 50 h for 2'-OH-TCC, 3'-OH-TCC and 6-OH-TCC, DHC and 20 h for 2'-O-Gluc-TCC (Fig. 4D).

A simultaneous increase in the water concentration with decrease in fish tissue concentration of these metabolites was observed (Fig. 4B) except for 2'-O-Gluc-TCC where the metabolite concentration in the water did not correspond with its elimination from the fish tissue. After a sharp increase in the water concentration between 5 min and 24 h post exposure up to  $708 \pm 0.05$  pM, the concentration decreased (data not shown). The semi quantitative evaluation of the peak areas in tissue and water samples of 3'-O-Gluc-TCC and 6-O-Gluc showed a similar time course as 2'-O-Gluc-TCC. These findings may be explained by a degradation of the glucuronic acid conjugates in the water. The concentration of DCC (impurity 0.7% of 20  $\mu\text{g/L}$  TCC during exposure, see section 2.1) in fish tissue declined in a similar manner over the post incubation time as TCC (Supplementary data, Fig. S1B) with a  $t_{1/2}$  of less than 1 h. Moreover, the semi quantitative monitored elimination of the tentatively identified metabolite OH-DCC was comparable to the hydroxylated TCC metabolites (Fig. 4, Supplementary Data, Fig. S1–2).

## 4. Discussion

This study demonstrates for the first time, that TCC bio-concentrates in fish. Medaka fish absorb TCC from water with a log BCF of  $2.86 \pm 0.05$  indicating a moderate bioaccumulation. This value is lower than log BCF value of 3.2–3.4 described for algae and snails (Coogan et al., 2007; Coogan and La Point, 2008). It is also significantly lower than the theoretical BCF calculated from the *n*-octanol/water partition coefficient ( $K_{OW}$ ). The correlation of BCF in fish and  $K_{OW}$  is expressed by Eq. (1) (Veith et al., 1980):

$$\log \text{BCF} = 0.76 \log K_{OW} - 0.23 \quad (\text{equation 2})$$

Using a calculated log  $K_{OW}$  of 4.9 for TCC and 4.3 for DCC (Sapkota et al., 2007) a theoretical log BCF of 3.5 for TCC and 3.0 for DCC results. The observed lower bioconcentration can be explained by the observed rapid metabolism of TCC by medaka fish. The metabolites detected in fish tissue were 2'-O-Gluc-TCC, 2'-O-SO<sub>3</sub>-TCC, 2'-OH-TCC, 3'-OH-TCC and 6-OH-TCC. This formation of sulfate and glucuronic acid conjugates of the oxidative metabolites shows that medaka fish larvae contain enzymes for both phase I and phase II metabolism. The presence of these enzymes in medaka are consistent with

previous findings (James et al., 1994). The primary formation of conjugates of 2'-OH-TCC, the pattern of TCC metabolites in the fish tissue is similar to the metabolites reported in mammalian plasma (Birch et al., 1978). Moreover, the dehalogenated and hydroxylated metabolite DHC, which was recently predicted *in vitro* (Baumann et al., 2010), was also found in significant amounts. Interestingly, neither *N*- and *N'*-glucuronides of TCC were detected in water or in fish tissue. These metabolites formed by direct conjugation of TCC are the major metabolites in human and monkey urine but not in rats (Birch et al., 1978; Schebb et al., 2011b). The data indicate that TCC undergoes a similar oxidative metabolism in fish as in other mammals, with a predominant formation of *ortho*-hydroxylated metabolites, followed by their conjugation with sulfate and glucuronic acid. In contrast, *N*-glucuronidation seems to be highly species dependent and only occurs in few mammalian species.

After transferring the medaka into fresh water, TCC was quickly eliminated from fish tissue ( $t_{1/2} = 1$  h). This elimination was almost exclusively mediated by metabolism and only 1% of the TCC accumulated remained after 48 h in the fish tissue or is detected in the aquarium water (Fig. 4C). The metabolites showed a longer half life of 20–50 h. However, 48 h post-transfer to clean water, the predominant amount (>90%) of TCC had been eliminated as metabolites into the water. It should be noted, that this is a relative distribution which was calculated based on the metabolites quantified by the method. Other forms of metabolites, such as the detected but not quantified 3'-*O*-Gluc-TCC and 6'-*O*-Gluc-TCC, would shift the distribution further towards the metabolites.

## 5. Conclusion

In this study, absorption, metabolism and elimination of TCC were investigated in fish for the first time. Our results, with medaka fish larvae as model organism, show that TCC moderately concentrates in fish with log BCF of  $2.86 \pm 0.05$ . An exposure level of 20 µg/L TCC, which is only slightly higher as TCC concentrations found in surface water of up to 5 µg/L (Halden and Paull, 2004, 2005), resulted in a maximal fish tissue concentration as high as 35 µmol/kg (11 mg/kg). However, absorbed TCC was rapidly eliminated by the fish ( $t_{1/2} = 1$  h), which is unexpectedly rapid for a compound with a predicted  $K_{OW}$  of 4.9 and three chlorine substituents. The rapid elimination was facilitated by fast metabolism to the same metabolites previously reported for mammals. Because of the rapid elimination, it is unlikely, that short term exposure of fish to TCC will result in accumulation of this antibacterial.

Nevertheless, during longer exposure, TCC bioconcentrates to a steady state according to its BCF. The resulting TCC tissue concentrations may elicit biological effects, such as inhibition of sEH. Given that mammal and fish sEH are similarly inhibited by urea derivatives (Newman et al., 2001), a tissue level within the range of the  $IC_{50}$  (human  $24 \pm 5$  nM, rat  $18 \pm 1$  nM (Schebb et al., 2011b)) is reached according to the determined log BCF of 2.86 by water concentrations as low as 35 pM (11 ng/L). This concentration is well below frequently detected levels of TCC in surface waters (Halden and Paull, 2004, 2005). Furthermore, toxic effects on the fish might be caused by the formation of reactive TCC metabolites, through adduct formation with cellular macromolecules. It has been recently shown that oxidative metabolism can lead to reactive quinone imine species, which covalently bind to GSH and proteins (Baumann et al., 2010). Though the precursor of these reactive compounds, 2'-OH-TCC was the predominantly formed metabolite in medaka fish, no signs of acute toxicity were observed during and after 24 hours incubation with 20 µg/L TCC. The absence of acute effects might be related to the rapid conjugation (phase II metabolism) of 2'-OH-TCC to 2'-*O*-Gluc-TCC. Based on our data it is not possible to evaluate whether reactive metabolites might cause observable toxic effects. It can also not

be determined if measured bioconcentration of TCC at environmental exposure levels could result in endocrine disruption in aquatic species. In rats, the ability of oral administration of 0.25 g/100 g TCC in the diet for 10 days (corresponding to a daily oral dose of about 0.25 g TCC / kg bodyweight) causes endocrine effects (Chen et al., 2008). However, the systemic TCC dose of the rats was not determined, which makes it impossible to compare the levels to the concentration observed in medaka fish larvae. Because it is also unclear if the testosterone-induced androgen receptor activation of TCC is similarly influenced in fish as previously seen in rats, no conclusion can be made based on our results concerning the possibility that endocrine effects might occur in fish. Ongoing research at the Aquatic Health and Superfund Research Program at UC Davis and the Institute of Toxicology at the University for Veterinary Medicine, Hannover, Germany seeks to provide answers to these questions and thus evaluate if TCC, as well as other persistent anthropogenic chemicals in the environment, have observable effects that are cause for concern and therefore potential impacts on environmental or human health.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## List of abbreviations

<b>BCF</b>	Bioconcentration factor
<b>DCC</b>	diclocarban; 4,4'-dichloro carbanilide
<b>DHC</b>	3',4' dichloro-4'-hydroxy-carbanilide
<b>EPA</b>	Environmental Protection Agency
<b>ESI</b>	electro-spray ionization
<b>gluc</b>	glucuronyl
<b>I.S.</b>	Internal Standard
<b>K<sub>ow</sub></b>	<i>n</i> -octanol-water-coefficient
<b>LC</b>	liquid chromatography
<b>LOD</b>	limit of detection
<b>MS</b>	mass spectrometry
<b>PCP</b>	personal care product
<b>P450</b>	Cytochrome P-450 monooxygenases
<b>sEH</b>	soluble epoxide hydrolase
<b>SPE</b>	solid phase extraction
<b>SRM</b>	selected reaction monitoring
<b>TCC</b>	triclocarban

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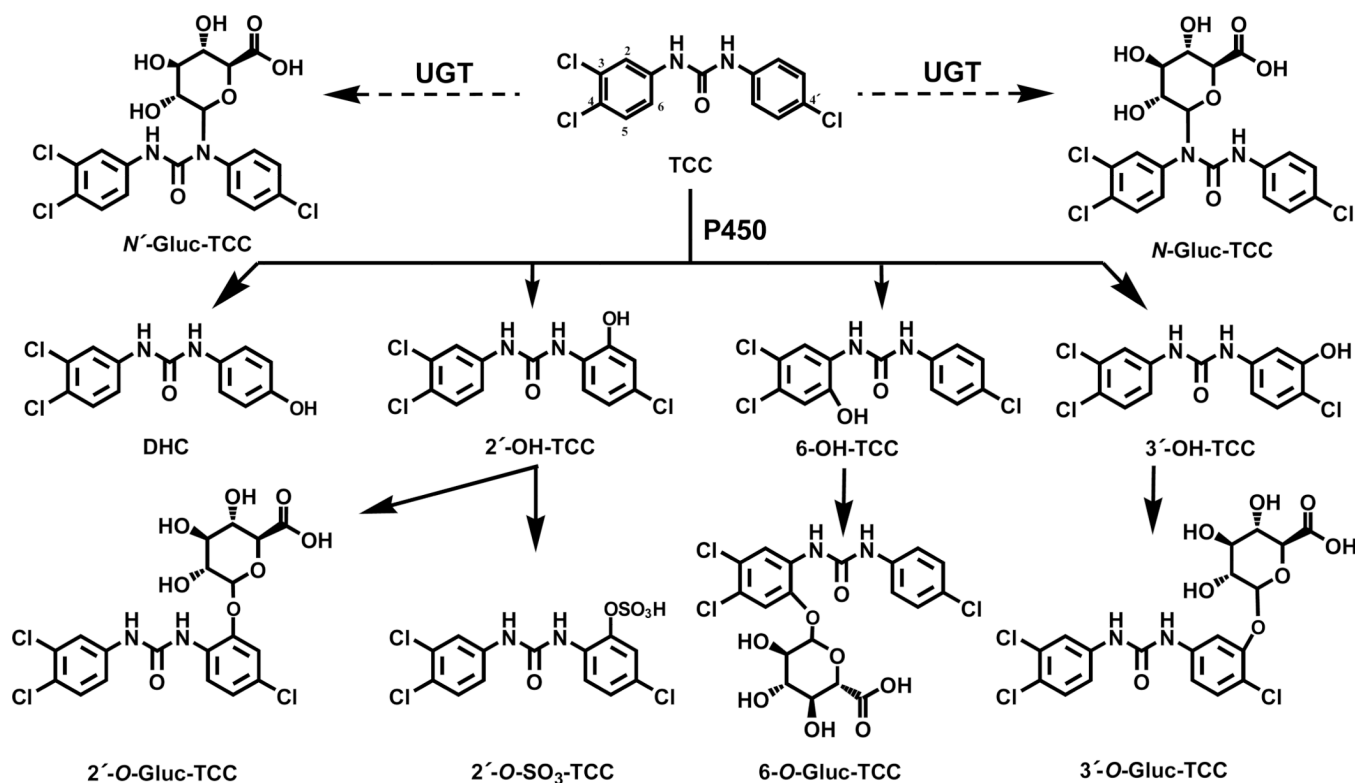


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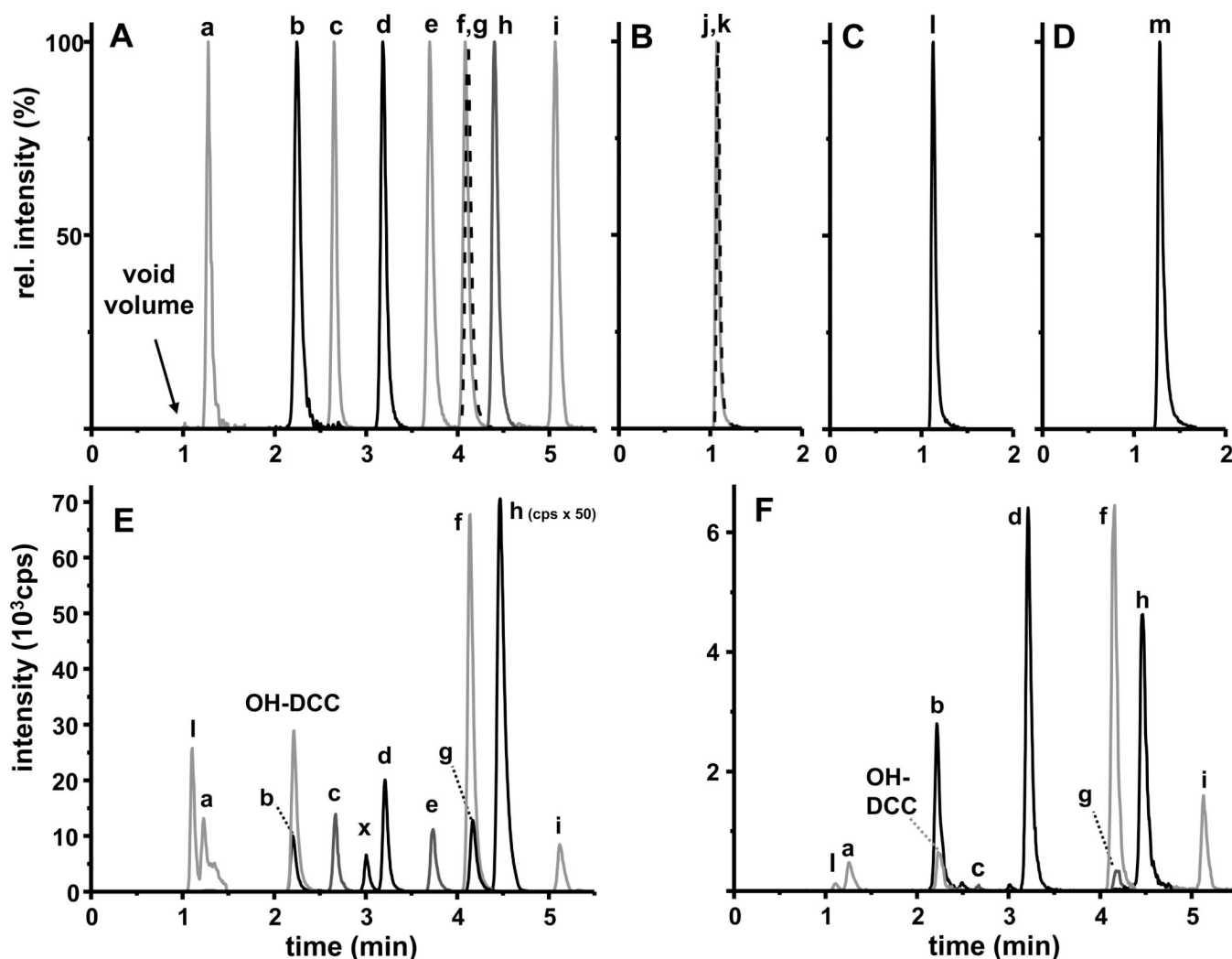
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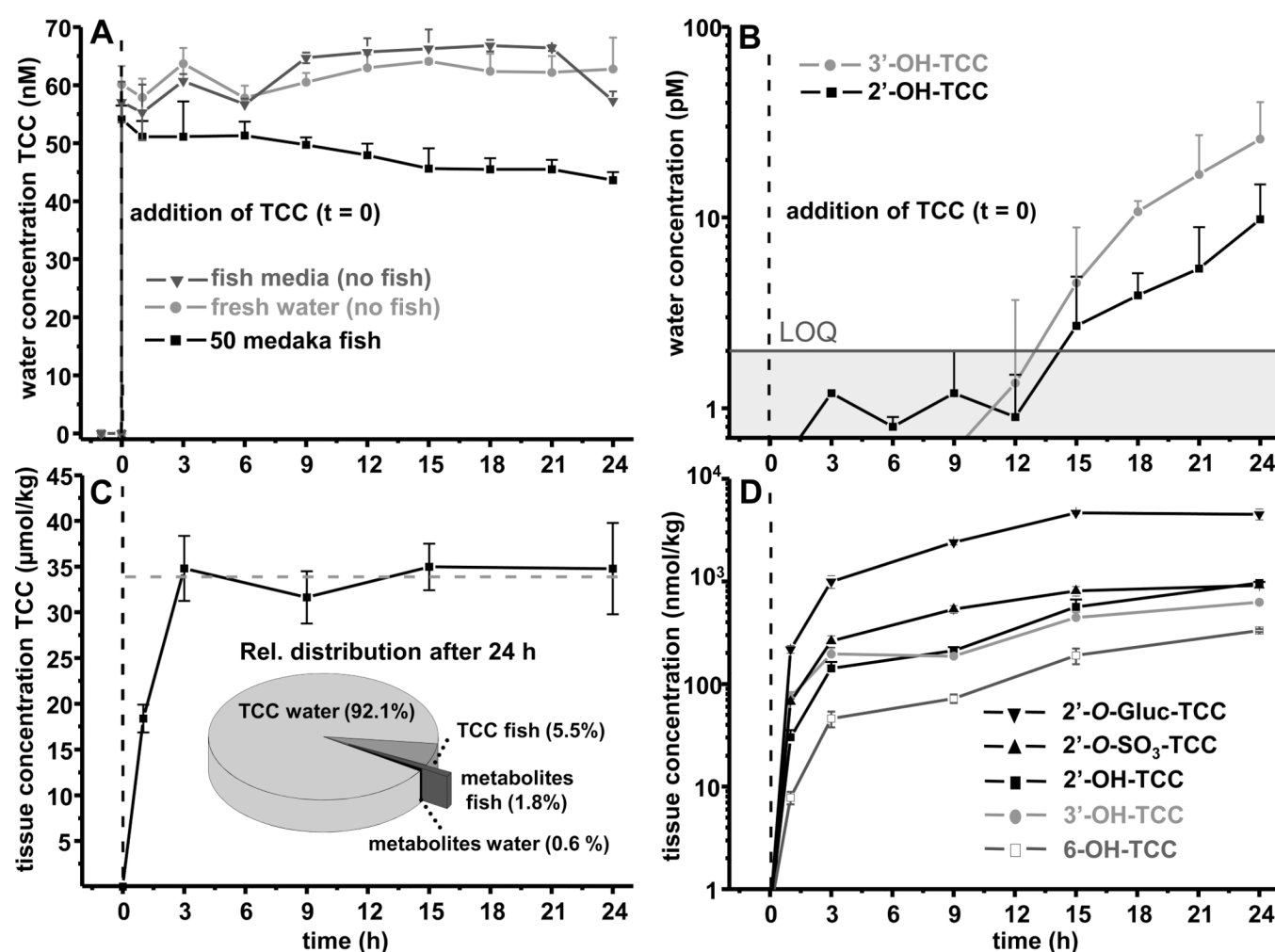
**Fig. 1.**

Scheme of TCC metabolism. Cytochrome P450 monooxygenases leads to the hydroxylated metabolites 2'-OH-TCC, 3'-OH-TCC, 6-OH-TCC and additionally to a dechlorinated hydroxylated product (DHC) (Baumann et al., 2010; Birch et al., 1978). Direct glucuronidation of TCC by UGT leads to *N*-glucuronides accounting for the major metabolites in human urine (Schebb et al., 2011b). The glucuronides of 2'-OH-TCC, 3'-OH-TCC, 6-OH-TCC are the major metabolites in mammalian bile and the sulfate conjugate (2'-O-SO<sub>3</sub>-TCC) is the dominating metabolite in human plasma (Birch et al., 1978). Solid arrows show pathways found in medaka and in mammalian species while dashed arrows show pathways only found in mammals.



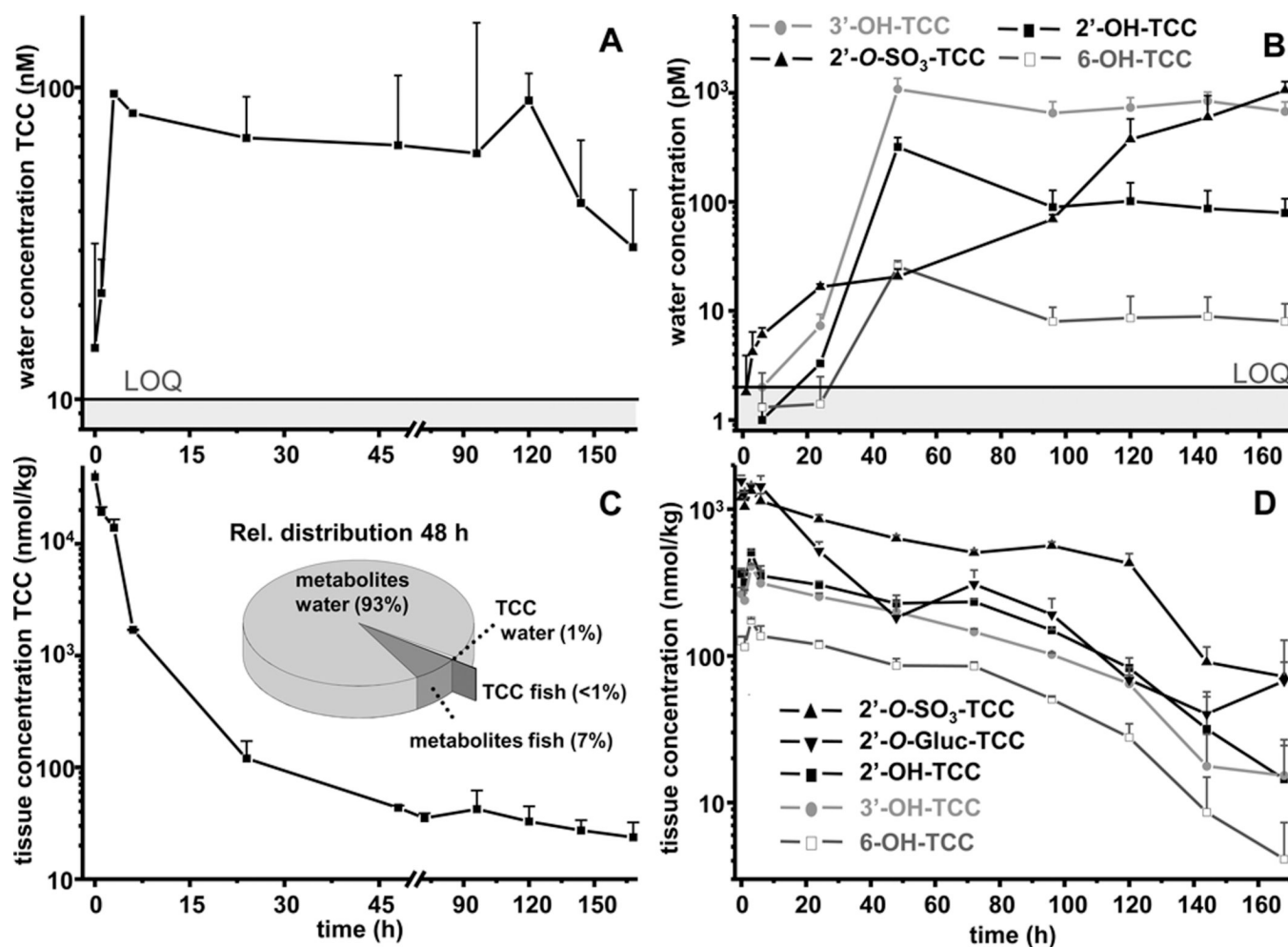
**Fig. 2.** Separation efficiency of the online-SPE-LC-MS/MS system. **A** Normalized SRM chromatograms of an injection of 100  $\mu$ l of a 0.1 nM standard solution: a 2'-O-Gluc-TCC, b DHC c 2'-O-SO<sub>3</sub>-TCC, d 3'-OH-TCC, e DCC, f and g 2'-OH-TCC and 6-OH-TCC (co-eluting), h TCC and i 3'-Cl-TCC. **B** Injection of a urine sample from a TCC exposed human containing *N*-Gluc-TCC (j) and (k) *N*'-Gluc-TCC. **C** and **D** 3'-O-Gluc-O-TCC (l) and 6-O-Gluc-O-TCC (m) generated by incubations with human liver microsomes **E** SRM chromatogram of medaka fish homogenate after 24 hours exposition with TCC (20  $\mu$ g/L). **F** water sample 48 hours post exposure.





**Fig 3.**

Accumulation and metabolism of TCC by medaka larvae during 24 hours exposure with a water concentration of 20  $\mu\text{g/L}$  TCC (63 nM). Time course of **A**: TCC water concentration (300 mL) with medaka larvae ( $n=50$ ) and used media and fresh water as control. **B**: Metabolite water concentration **C**: TCC concentration in fish tissue **D**: Metabolite concentration in fish tissue. All concentrations are shown as mean and of three independent incubations. No metabolites were detected in water and fish samples from control experiments. The relative distribution of TCC after 24 hours is summarized in the insert in panel C.

**Fig 4.**

Elimination and metabolism of TCC: medaka larvae (n= 50) were exposed 24 hours to TCC (20 µg/L) and transferred into 300 mL fresh water (t=0). Time course of **A**: TCC water concentration. **B**: Metabolite water concentration **C**: TCC concentration in fish tissue **D**: Metabolite concentration in fish tissue. All concentrations are shown as mean and of three independent incubations. The insert in panel C show the relative distribution of TCC and its metabolites after 48 h.